Fast and High-Resolution Reversed-Phase Separation of Synthetic Oligonucleotides

High-pH-stable, superficially porous particle columns for LC/UV and LC/MS

Application Note

Biologics and Biosimilars

Authors

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Introduction

Synthetic DNA- and RNA-based oligonucleotides are among many successful biotherapeutic drugs to treat many different kinds of illnesses. They are synthesized in a multistep process. Although coupling efficiencies are high, the overall yield of oligos decreases as the number of cycles increases, with failure in coupling with single (N-1) and double (N-2) deletions as the major impurities. To ensure drug potency, and to reduce the potential for drug interactions, a high-purity product is required, therefore, analyzing the purity of the products is important. There are many methods used for analyzing oligonucleotides. One of the most common involves anion-exchange chromatography. This can provide high resolution, but the separation often takes a long time. Moreover, due to its solvent systems, eluting oligonucleotides with high salt concentrations make this method incompatible with mass-spectrometry. This makes the identification of oligonucleotides and their impurities very cumbersome.

In this application note, we demonstrate fast and high-resolution separation and identification of a number of de-protected (removal of the dimethoxytrityl group (DMT)) DNA and RNA oligonucleotides, using Agilent AdvanceBio Oligonucleotide columns. These are high-pH-stable reversed-phase columns packed with superficially porous Poroshell particles. The particles have a porous outer layer and solid core that limit diffusion distance, and, in combination with a very narrow particle size distribution, improve separation speed and chromatographic efficiency. With 2.7 µm diameter particles and 600 bar pressure rating, the columns are easily operated on HPLC and UHPLC instruments.

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The Poroshell particles are chemically modified using proprietary technology that makes them very resistant to high-pH mobile phases up to pH 11.0. The endcapped C18 bonded phase and 100Å pore diameter provide excellent selectivity for oligonucleotides. The data here include the separation and identification of DNA and RNA oligonucleotides using two popular mobile phase gradients. The eluents are volatile and MS compatible. One contains triethylammonium acetate (TEAA) commonly used in LC/UV separations, and the other contains hexafluoroisopropanol and triethylamine (HFIP:TEA), commonly used in LC/MS analysis for oligonucleotides. The LC/MS data also demonstrate excellent mass accuracy and provide sequence information of some oligonucleotides. Results are compared to the separation on a totally porous, hybrid particle column.

Materials and Methods

The column was an AdvanceBio Oligonucleotide, 2.1 × 50 mm (p/n 659750-702). Acetonitrile, methanol, TEAA, HFIP, and TEA were purchased from Sigma-Aldrich, Corp.

The DNA samples were DNA oligonucleotides (Agilent Oligonucleotide Ladder Standard, p/n 5190-9029) (Table 1). The 23-mer RNA used in the column comparison and the 25-mer DNA used for the MS data are from NASD, Boulder.

The RNA samples were RNA oligonucleotides (Agilent Oligonucleotide Resolution Standard, p/n 5190-9028) (Table 2).

LC/UV conditions

Mobile phase:	A) 100 mM TEAA in water B) 100 mM TEAA in acetonitrile
Flow rate:	0.6 mL/min (or other flow rates)
Gradient:	See chromatogram
Stop time:	See chromatogram
Post run:	5 min
Column temperature:	65 °C
Sample:	See chromatogram
Injection:	See chromatogram
Detection:	UV at 260 nm

LC/MS friendly conditions

Mobile phase:	A) HFIP:TEA (400 mM:15 mM) in water B) methanol:mobile phase A (50:50)
Flow rate:	0.4 mL/min
Gradient:	30 to 40% B in 0.5 min, 40 to 70% B in 5 min
Sample:	25-mer DNA
Temperature:	65 °C
Detection:	MS, or UV at 260 nm
Min range:	400 *m/z*
Max range:	1,700 *m/z*
Scan rate:	3.00 spectra/s
Ion polarity:	-ve
VCap:	3,500
Nozzle voltage:	1,000 V
Fragmentor:	200

Instruments

- Agilent 1290 Infinity LC
- Agilent 6530 Accurate Mass Q-TOF LC/MS

Table 1. DNA-oligonucleotide characteristics.

Sequence	Sequence	Purity guarantee	Amount (nmole)
15-mer	TTTTT TTTTT TTTTT	>85%	2
20-mer	TTTTT TTTTT TTTTT TTTTT	>85%	2
25-mer	TTTTT TTTTT TTTTT TTTTT TTTTT	>85%	2
30-mer	TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT	>85%	2
35-mer	TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT	>85%	2
40-mer	TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT	>85%	2

Table 2. RNA-oligonucleotide characteristics.

Sequence	Sequence	Purity guarantee	Amount (nmole)
14-mer	rCrArCrUrGrArArUrArCrCrArArU	>85%	2
17-mer	rUrCrArCrArCrUrGrArArUrArCrCrArArU	>85%	2
20-mer	rUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU	>85%	2
21-mer	rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU	>85%	4

Results and Discussion

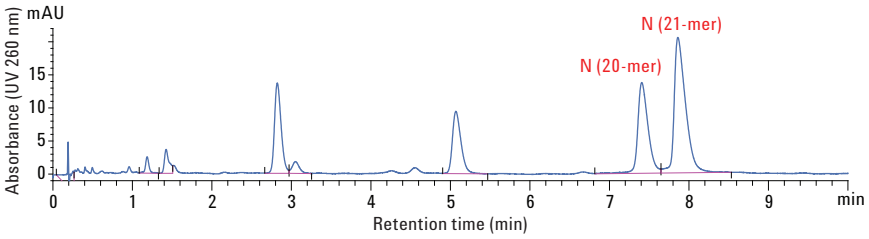
Resolving N and N-1 oligonucleotides

Separation of RNA oligonucleotides

The oligonucleotide resolution standard contains four RNA oligonucleotides ranging from 14- to 21-mer, and is designed for verification of instrument and column performance (lot-to-lot) for analysis of synthetic oligonucleotides. Figure 1 illustrates the separation power of the AdvanceBio Oligonucleotide column for this standard. All peaks were separated in under nine minutes; the peaks were sharp and well resolved. The N and N-1 RNA oligonucleotides (21- and 20-mer) were separated close to baseline. These data suggest that the column was very capable of resolving a main oligonucleotide from its impurities.

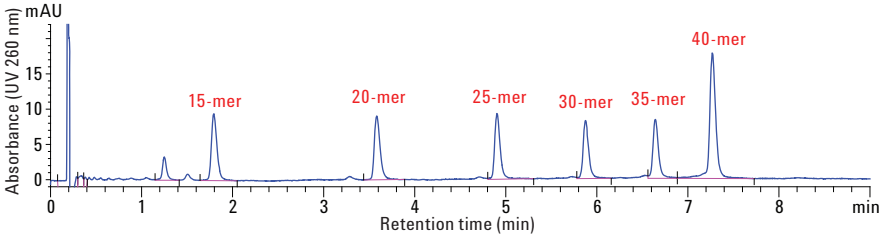
Separation of DNA oligonucleotides

Another demonstration of the resolving capacity of complex samples by AdvanceBio Oligonucleotide is provided by the separation of an Oligonucleotide Ladder Standard that contains six DNA oligonucleotides, ranging from 15- to 40-mer. Figure 2 demonstrates that the separation of all six oligonucleotides was completed in less than eight minutes. All the peaks were separated with baseline resolution. The 15-mer DNA oligonucleotide began to elute in as little as two minutes, and the 20- and 25-mer were eluted at four and five minutes, respectively. These data indicate that AdvanceBio Oligonucleotide columns are suitable for high throughput use in separating oligonucleotides.



Gradient: 10 to 14% B in 10 min
Stop time: 11 min
Sample: Agilent Oligonucleotide Resolution Standard
14-mer, 17-mer, 20-mer, 21-mer RNA
(p/n 5190-9028)
Injection: 10 µL

Figure 1. Separation of Agilent Oligonucleotide Resolution Standard using an Agilent AdvanceBio Oligonucleotide column.



Gradient: 6 to 8% B in 12 min
Stop time: 13 min
Sample: Agilent Oligonucleotide Ladder Standard
15-mer, 20-mer, 25-mer, 30-mer, 35-mer,
40-mer DNA (p/n 5190-9029)
Injection: 0.5 µL

Figure 2. Separation of Agilent Oligonucleotide Ladder using an Agilent AdvanceBio Oligonucleotide column.

Mass-spectrometry compatibility

To confirm the identity and purity of the newly produced and replica-production lots of oligonucleotides, mass spectrometry is used for the analysis. Figure 3 shows the MS results from a single DNA oligonucleotide of 25-mer. The AdvanceBio Oligonucleotide column generated high chromatographic resolution for the 25-mer DNA oligo peak and its impurities in only about three minutes. The UV and TIC traces, with the expansion view of the baselines, were recorded and reported. The expansion of baseline resolution of the main peak and N-peaks indicated that the two traces were very similar, and that they closely lined up. The compatibility between two types of detection showed that the UV trace had sufficient sensitivity to be used for ladder runs to determine the main peak and its impurities (N minuses peaks) without using the MS again, once the MS data was fully established. Figure 4 shows the deconvoluted data from the TIC traces with fully labeled peaks and Table 3 shows the accurate masses and percentages of structures in the analysis from the main peak of the 25-mer DNA oligonucleotide and its impurities. The sum of this table demonstrated that the column provides 100% recovery.

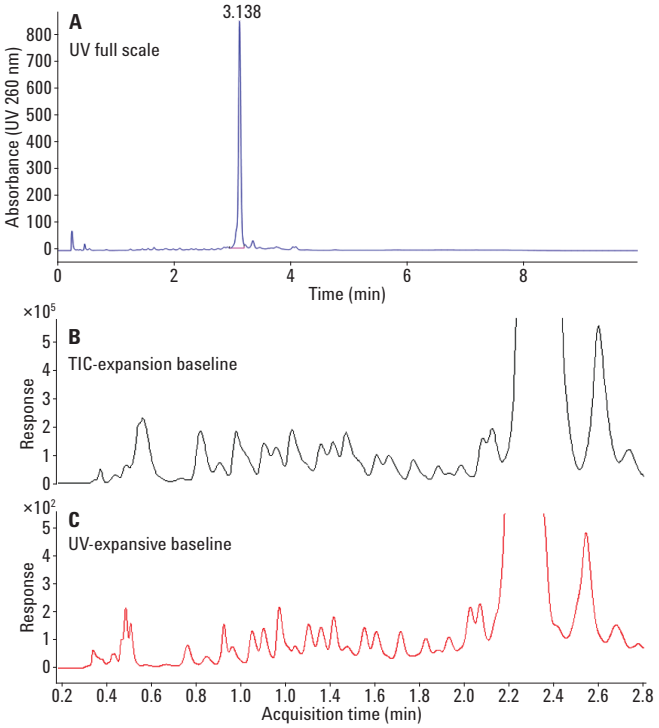


Figure 3. Separation of 25-mer DNA oligonucleotide using MS-compatible solvent.

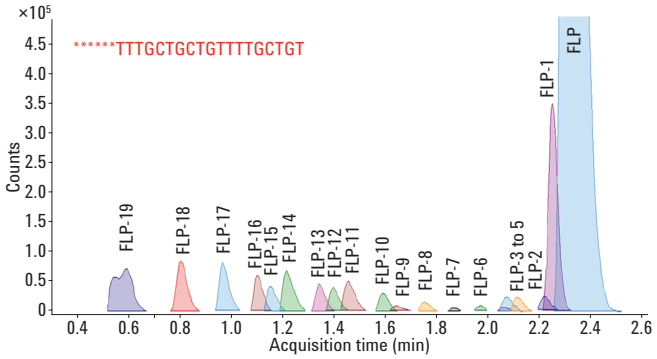


Figure 4. Deconvoluted data from TIC of a 25-mer DNA oligonucleotide separated by the Agilent AdvanceBio Oligonucleotide column.

Table 3. MS analysis and recovery.

Peak	Response	%
FLP	5089897	44.33
FLP-1	1656225	14.42
FLP-2	304129	2.65
FLP-3	303848	2.65
FLP-4	218243	1.90
FLP-5	113062	0.98
FLP-6	104555	0.91
FLP-7	110327	0.96
FLP-8	134341	1.17
FLP-9	134080	1.17
FLP-10	186947	1.63
FLP-11	358833	3.12
FLP-12	251690	2.19
FLP-13	272844	2.38
FLP-14	416306	3.63
FLP-15	238205	2.07
FLP-16	304333	2.65
FLP-17	403038	3.51
FLP-18	459344	4.00
FLP-19	422518	3.68
Sum	11482765	100

Column comparison

Superficially porous particles are known to have faster mass transfer due to the nature of the particles' construction, that is, a solid core surrounded by a thin porous layer that allows shorter distances for diffusion into and out of the porous structure. Figure 5 compares separations of 23-mer RNA oligonucleotide between the AdvanceBio Oligonucleotide with 2.7 µm particles and a totally hybrid porous 2.1 × 50 mm column with 1.7 µm totally porous hybrid particles. Data indicate that the peak width of the 23-mer RNA oligonucleotide on the AdvanceBio Oligonucleotide column was narrower than that generated by the 1.7 µm totally hybrid porous particle C18 column. This supports the fact that shorter distances for diffusion into and out of the superficially porous stationary phase result in faster mass transfer, producing peaks with higher resolution. The 2.7 µm particle size of the AdvanceBio Oligonucleotide column permitted operation at a lower backpressure compared to the 1.7 µm totally hybrid particle C18 column, 108 bar versus 292 bar at 0.4 mL/min, respectively. Therefore, AdvanceBio Oligonucleotide columns are compatible with 600 bar HPLC systems, as well as 1200 bar HPLC systems for fast and high-speed separation.

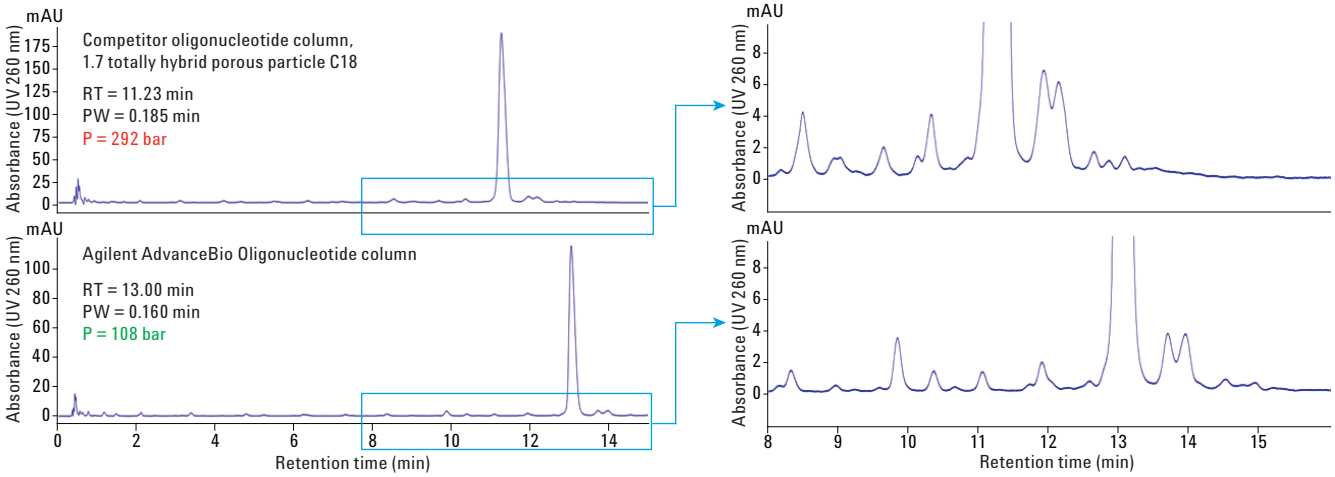
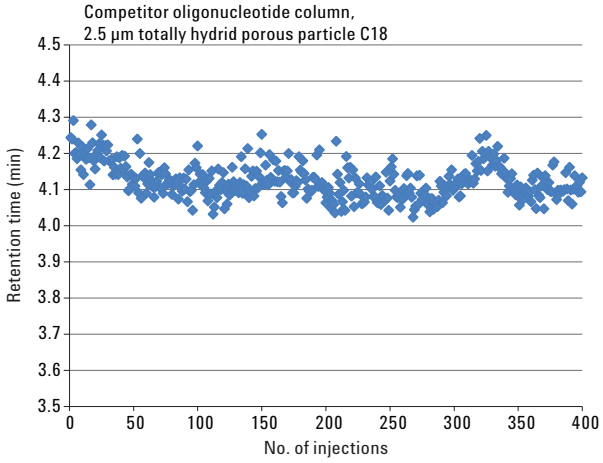
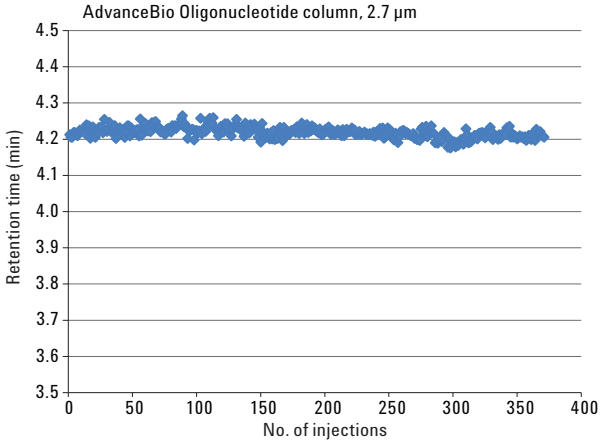


Figure 5. Comparing the 25-mer DNA oligonucleotide separation on an AdvanceBio Oligonucleotide 2.7 µm column versus a 1.7 µm totally hybrid porous particle C18 column using HFIP:TEA in the mobile phases.

Column stability

Figure 6 shows column stability and comparison data between the AdvanceBio Oligonucleotide column and the 2.5 µm totally hybrid porous particle C18 column, both with 2.1 × 50 mm dimensions. Data were collected from ~400 consecutive injections of 25-mer DNA oligo at 65 °C. The peak retention time was recorded and showed that the AdvanceBio Oligonucleotide column generated stable, nearly unchanged, and highly reproducible peak retention times, slightly better than that of the competitor oligonucleotide column, 2.5 µm totally hydrid porous particle C18. These stability data suggest that the AdvanceBio Oligonucleotide column has a long and comparable lifetime to the 2.5 µm totally hybrid porous column.





Flow rate:	0.69 mL/min
Gradient:	7 to 11% B in 5 min
Run time:	8.5 min
Sample:	25-mer RNA
Injection:	1 µL of 0.5 mg/mL

Figure 6. Column stability study.

Conclusions

The AdvanceBio Oligonucleotide column, 2.7 µm, with highly stable pH superficially-porous particles is designed for separating deprotected RNA and DNA oligonucleotides of different sizes with speed and high resolution. The columns can be used with both LC/UV and LC/MS mobile phases containing high pH iron-paring agents such as TEAA and HFIA:TEA (>pH 8.0), the ideal pH for analyzing oligonucleotides. The AdvanceBio Oligonucleotide column was also shown to have the chemistry that delivers exceptional sample resolution and superior column lifetime. With 2.7 µm particles, the columns are compatible with 600 bar HPLC, and 1,200 bar UHPLC systems. AdvanceBio Oligonucleotide columns are packed in various column dimensions, which provide many selection options to meet various demands of isolation and analysis for both DNA- and RNA-based oligonucleotides.

For More Information

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